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Influence of stromal cells on lymphocyte adhesion and migration on endothelial cells

Helen M. McGettrick, PhD, **Chris D. Buckley, PhD**, **G. Ed Rainger, PhD**, and **Gerard B. Nash, PhD**
Centre for Cardiovascular Sciences and Centre for Immune Regulation, The Medical School, University of Birmingham, Birmingham B15 2TT, UK

Abstract

Methods are described for analysing adhesion and migration of isolated lymphocytes on endothelial cell monolayers which have been co-cultured with different stromal cells, with or without additional cytokine treatment. The different cells types are grown on opposite sides of 3.0 or 0.4μm pore filters, depending on whether migration through the whole construct is to be analysed, or adhesion to the endothelial cells alone. Assays may be 'static' or filters can be incorporated into flow chambers so that cell behaviour can be directly observed under conditions simulating those in vivo. In general, by choice of method, one can evaluate efficiency of attachment, and ability of cells to migrate across the endothelial monolayer, through the filter and through the stromal cell layer. Fluorescence microscopic examination of fixed filters can be used e.g., to ascertain whether lymphocytes are retained by stromal cells. In general, static assays have the higher throughput and greatest ease of use, while the flow-based assays are more physiologically-relevant and allow detailed recording of cell behaviour in real time.

Keywords

Lymphocyte; endothelial cells; fibroblasts; smooth muscle cells; stromal cells; adhesion; migration; cytokines; cell culture; co-culture

1. Introduction

Leukocyte recruitment is regulated by the local haemodynamic and stromal environments (1). Stromal cells such as fibroblasts or smooth muscle cells (SMC) may influence the normal physiological responses of endothelial cells (EC), while changes in their phenotypes may be associated with chronic inflammatory disorders. For instance, we found that culture of SMC in the secretory state with EC, cause marked augmentation of the capture of all types of flowing leukocytes in response to tumour necrosis factor-α (TNF) (2). In addition, fibroblasts from inflamed tissue (the synovium of patients with rheumatoid arthritis) directly induced adhesion of neutrophils and lymphocytes when cultured with EC (3,4). Such studies indicate that stromal cells can contribute to tissue- or vessel-specific patterns of leukocyte recruitment, may modulate inflammatory responses in general or influence the development of disease at specific sites. Thus experimental models in which one can study how lymphocyte adhesion and migration are modified by stromal cells have a variety of potential uses.

All correspondence to: Prof. G.B. Nash, Department of Physiology, The Medical School, The University of Birmingham, Birmingham B15 2TT, UK, Tel: 00 44 (0)121 414 3670, Fax: 00 44 (0)121 414 6919, g.nash@bham.ac.uk.

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Here we describe several such models. In general they rely on culturing EC on one side of a porous filter and the stromal cells on the other, with or without stimulation with cytokines. Subsequent assays of lymphocyte adhesion can be carried out after settling cells onto the endothelial surface for prolonged periods, or during perfusion of cells in suspension. If larger-pore filters (diameter ~3μm) are used, it is possible to follow lymphocyte migration through the two layers of cells. The 'static' assays generally quantify how many cells migrate through the co-culture construct, but filters can be cut out and studied microscopically to assess whether cells are retained in the stromal layer. Practically, in flow systems, we have designed chambers for fluorescence microscopy which hold smaller filter inserts (24-well), and used them to quantify the capture process and whether cells become activated and stabley adherent or not (5). We have also used larger 6-well inserts, cut the filters out and incorporated them in chambers designed for phase-contrast microscopy (6), so that we can follow cells binding and then migrating through the endothelial monolayer, across the filter and into the stromal layer in real time.

2. MATERIALS

2.1 Blood cell isolation

1. K_2-EDTA in 10ml tubes (Sarstedt, Numbrecht, Germany).
2. Histopaque 1077 (H1077) (Sigma-Aldrich, Poole, UK).
3. PBSA: phosphate buffered saline with 1mM Ca^{2+} and 0.5mM Mg^{2+} (PBS Gibco, Invitrogen Ltd., Paisley, UK), with 0.15% (w/v) bovine albumin (dilute from 7.5% culture-tested solution; Sigma) and 5mM glucose.
4. *M199-BSA:* Medium 199 (M199 - Gibco) supplemented with 0.15% (w/v) bovine albumin (M199BSA).
5. 2% glutaraldehyde (Cowley, Oxford, UK) diluted in 1/3 strength PBS to be isotonic.
6. Fluorescent nuclear stain, bisbenzamide (stock at 1mg/ml; Sigma).

2.2 Culture of endothelial cells

1. M199 supplemented with gentamycin sulphate (35μg/ml), human epidermal growth factor (10ng/ml; Sigma E9644), and fetal calf serum (FCS) (20% v/v heat-inactivated) (all from Sigma). Adding hydrocortisone (1μg/ml, from 10mg/ml stock in ethanol; Sigma) improves growth if going beyond 1st passage.
2. Bovine skin gelatin (Type B, 2% solution, culture tested; Sigma).
3. Collagenase (type IA; Sigma) stored at −20°C at 10mg/ml in PBS. Thawed and diluted to 1mg/ml with M199 for use.
4. Autoclaved cannulae and plastic ties (electrical).
5. EDTA solution (0.02%, culture tested; Sigma).
6. Trypsin (2.5mg/ml; Sigma)
7. 70% (v/v) ethanol or industrial methylated spiritis.
8. Tumour necrosis factor-α (TNF) (Sigma) and interferon-γ (IFN; Peprotech Inc. London, UK), stored in small aliquots at −80°C.

2.3 Culture of stromal cells

1. Fibroblast complete medium: RPMI 1640 medium (Gibco) supplemented with 1X MEM-non-essential amino acids (stock was at 100x), 1mM sodium pyruvate, 2mM L-glutamine, 100U/ml penicllin, 100μg/ml streptomycin, and FCS (10% v/v heat-inactivated) (all from Sigma).
2. Promocell smooth muscle cell (SMC) medium supplemented with gentamycin sulphate (12.5μg/ml), amphotericin B (12.5ng/ml), human epidermal growth factor (10ng/ml), basic fibroblast growth factor (2ng/ml), dexamethasone (0.4μg/ml) and FCS (5% v/v heat-inactivated) (basal medium and all additional supplements from Promocell, Heidleberg, Germany) .
3. Sterile dissecting scissors, scalpel and forceps.
4. EDTA solution (0.02%, culture tested; Sigma).
5. Trypsin (2.5mg/ml; Sigma).
6. 70% (v/v) ethanol or industrial methylated spiritis.
7. Dimethylsulphoxide hybrid-max (DMSO; Sigma).

2.4 Surfaces for endothelial and stromal cell culture for assays

1. *Cell culture inserts:* High-density 0.4μm or low-density 3.0μm pore polycarbonate filter inserts in 24-, 12- or 6-well format (referred to as filters in future text) with matching culture plates (BD Pharmingen, Oxford, UK).

2.5 Flow-based adhesion assay

1. *Parallel-plate flow chamber for fluorescence microscopy - for use with intact 24-well transwell filters* (5) (Fig 1): A glass coverslip (5.5 × 2.6mm). A non compressible silicon gasket, 250μm thick, containing a 41 × 6mm slot which forms the flow channel. Specially designed chamber made up of two parallel plates held together with six screws (Wolfson Applied Technology Laboratory, University of Birmingham, Birmingham, UK). The lower plate has a machined receiving slot of a complementary size for the 24-well insert, along with inlet and outlet channels. The upper perspex plate has a machined slot to allow objective lens access and a shallow recess milled in it to receive the coverslip.
2. *Parallel-plate flow chamber for phase contrast microscopy - for use with cut out 6-well filters (6)* (Fig 2)*:* A glass coverslip (75 × 26mm; Raymond A. Lamb, Eastbourne, UK). A Parafilm gasket (75 × 26mm) containing a 20 × 4mm slot. Specially designed chamber made up of two perspex plates held together with six screws (Wolfson Applied Technology Laboratory, University of Birmingham, Birmingham, UK). The lower plate has a counter-sunk viewing slot cut in it, and a shallow recess milled in it to receive the coverslip, filter and gasket. The upper perspex plate has inlet and outlet holes positioned to match the flow channel formed by the gasket slot, allowing liquid to be perfused over the HUVEC. The depth of the flow channel is defined by the thickness of the gasket, which averages 133μm. The gasket is cut out from a sheet of parafilm using a rectangular aluminium template (75 × 26mm) containing a 20 × 4mm slot.
3. *Flow system* (Fig 3): Syringe pump with smooth flow (e.g., PHD2000 infusion/withdrawal, Harvard Apparatus, South Natic, MA, USA). Electronic 3-way microvalve with minimal dead volume (LFYA1226032H Lee Products Ltd., Gerrards Cross, Buckinghamshire, UK.) and 12 volt DC power supply. Silicon rubber tubing, internal diameter/external diameter (ID/OD) of 1/3mm and 2/4mm

(Fisher Scientific, Loughborough, UK). Three-way stopcocks (BOC Ohmeda AB, Helsinborg, Sweden). Sterile, disposable syringes (2, 5, 10ml Becton Dickinson, Oxford, UK) and glass 50ml syringe for pump (Popper Micromate; Popper and Sons Inc., New York, USA).

4. *Video-microscope*: Microscope with heated stage or preferably, with stage and attached flow apparatus enclosed in a temperature controlled chamber at 37°C, with phase contrast and fluorescence (UV) optics. Video camera (e.g., analogue Cohu 4912 monochrome camera with remote gain control), monitor and video recorder (e.g., time lapse, Panasonic AG-6730), or digital camera (e.g., Olympus U-CMAD3 QICAH) directly connected to computer (see below).

5. *Image analysis*: Computer with video capture card (if using video recordings) or input for digital cameras, and specialist software for counting cells, measuring motion etc. There are a range of commercial packages available, as well as image analysis software (NIH Image http://rsb.info.nih.gov/nih-image/) available free over the Internet. We currently use Image Pro software (Media Cybernetics).

3. METHODS

3.1 Leukocyte isolation (see Note 1)

1. Draw blood from the ante-cubital vein of normal human volunteers with a minimum of stasis, dispense into K_2EDTA tubes and mix gently but thoroughly.
2. Place 5ml H1077 in 10ml centrifuge tube.
3. Layer whole blood (5ml) from K_2EDTA tube on top.
4. Centrifuge at 800g for 30 minutes.
5. Retrieve the mononuclear cells from the top of the gradient, at the interface of plasma and H1077.
6. Wash cells twice in PBSA or M199BSA.
7. To deplete mononuclear cells of monocytes, place in culture dish for 30 minutes at 37°C for monocytes to sediment and adhere. Gently wash off enriched peripheral blood lymphocytes (PBL).
8. Count lymphocytes and dilute to desired concentration in PBSA or M199BSA or endothelial culture medium (see Note 2).
9. For fluorescence, pre-label cells with 1μg/ml bisbenzimide 15min in the dark (see Note 3).

[1]There are various methods for isolating lymphocytes from blood and section 3.1 describes a simple one that we use regularly. In the early stages, it is advisable also to test viability of preparations (e.g., ~99% viable judged with trypan blue) and purity. Lymphocytes prepared in this way will still have some monocyte contamination. Further purification of lymphocyte subsets can be made using immunomagnetics selection (e.g., Dynabeads, Dynal Biotech UK, Bromborough, UK; MACS, Miltenyi Biotec Ltd. Bisley, UK).
[2]When added to HUVEC, PBSA is sufficient to maintain viability in short assays for up to about an hour. However, PBSA is unable to maintain an intact HUVEC monolayer following 24 hours of culture as judged by visual observations and a decrease in electrical resistance across the monolayer. Lymphocytes can be suspended in endothelial culture medium, but in our experience M199+BSA provides a simpler medium without growth factors and FCS which maintains endothelial morphology and electrical resistance for 24h.
[3]We have analysed the effects of various dyes on the capture, adhesion and migration of neutrophils and lymphocytes (8,9). In our hands, bisbenzimide has the least effect on behaviour. In general, the activatory or damaging effects of fluorescent dyes depend on the duration for which the cells are illuminated. Ideally, illumination should be restricted to the time necessary to capture microscopic images, and kept as short as possible.

3.2 Isolation and culture of endothelial cells and stromal cells

There are various methods for culture of endothelial and stromal cells from different sources, and for the novice, it is probably best to start by buying cells and media from commercial suppliers. A variety of different endothelial cells, fibroblasts and smooth muscle cells are available (e.g. from Asterand; Clonetics; ECCAC; Promocell). Our current method for isolating and culturing human umbilical vein endothelial cells is given below, adapted from Cooke et al. (7).

3.2.1 Isolation and primary culture of HUVEC—

1. Place the cord on paper towelling in a tray and spray liberally with the 70% ethanol. Choose sections of about 3-4 inches that do not have any clamp damage. Each 3-4 inch piece of cord equates to 1 flask of primary cells.
2. Locate the two arteries and one vein at one end of the cord.
3. Cannulate the vein and secure the cannula with an electrical tie.
4. Carefully wash through the vein with PBS using a syringe and blow air through to remove the PBS.
5. Cannulate the opposite end of the vein and secure with electircal tie.
6. Inject collagenase (~10ml per 3-4 inches) into vein until both cannulae bulbs have the mixture in them.
7. Place the cord into an incubator for 15 min at 37°C.
8. Remove from the incubator and tighten the ties. Massage the cord for ~ 1 min.
9. Flush the cord through using a syringe and 10ml PBS into a 50ml centrifuge tube.
10. Push air through to remove any PBS, repeat this twice more (3 × 10ml).
11. Centrifuge at 400g for 5 min. Discard supernatant.
12. Resuspend the cells in ~1ml of culture medium and mix well with pipette
13. Make up to 4ml in complete medium.
14. Add cell suspension to a 25cm^2 culture flask.
15. Change medium after 2h, the next day and every subsequent two days. Cells should be confluent in about 3-7 days.

3.2.2 Isolation and culture of primary fibroblasts—Here we give the procedures for isolating dermal fibroblasts and below, for isolating arterial smooth muscle cells from umbilical arteries. The former would require a clinical link through which to obtain skin tissue e.g., from patients undergoing surgery.

1. Obtain tissue (e.g., ~1cm^3) in a sterile container on ice.
2. If tissue is bloody, wash first with RPMI alone, centrifuge at 300g for 5min and discard supernatant.
3. Place tissue into a sterile petri dish. Each 1cm^{3}piece of tissue will seed 4 flasks.
4. Using sterile scalpel, remove the fatty (yellow) tissue from the skin.
5. ‘Tease’ apart the grey skin tissue into fine strand-like remnants less than 1mm^3. It may be necessary to pipette on a small amount of medium if the tissue starts to dry out and become ‘sticky’.

6. Add ~0.25cm^3 of tissue into a 25cm^2 culture flask (T25 flask).
7. Add 7ml of fibroblast complete medium.
8. Incubate undisturbed at 37°C in 5% CO_2 for 3 weeks. (Allows time for the fibroblasts to grow out of the tissue.)
9. Change medium by aspirating out 2/3 of old medium and replacing it with fresh. During this time only change medium when it becomes yellowish (see Note 4).
10. Initial outgrowth of adherent cells is usually seen after 1-2 weeks. Confluence is normally reached after 3-6 weeks although this depends on tissue type and may vary between donors.

3.2.3 Isolation and culture of primary 'secretory' smooth muscle cells from umbilical artery—

1. Place a 2 inch section of umbilical cord in a sterile petri dish.
2. Locate the two arteries and one vein at one end of the cord.
3. Hold the cord with the sterile forceps.
4. Using sterile dissecting scissors cut along the vein (see Note 5).
5. Open the cord flat and locate the arteries.
6. Cut between the arteries so that they are separated from one another other.
7. Cut away all the extraneous tissue surrounding 1 artery. It is essential to remove all the surrounding tissue to prevent contamination.
8. Cut the artery into 0.5-1mm pieces.
9. Add 6-10 pieces to a T25 flask. (One artery can be split between 3 separate T25).
10. Add 5ml of Promocell SMC medium.
11. Incubate undistrubed at 37°C for 3 weeks. (Allows time for smooth muscle cells to migrate out of the artery).
12. Visualise under phase contrast microscopy: smooth muscle cell colonies should have formed. Culture can be continued until confluence is reached (see Note 6).

3.2.4 Dispersal of endothelial and stromal monolayers for passaging—

1. Rinse a flask containing a confluent primary monolayer of cells or smooth muscle cell colonies with 2ml EDTA solution.
2. Add 2ml of trypsin solution and 1ml of EDTA for 1-2 minutes at room temperature, until the cells became detached. Tap on bench to loosen.
3. Add 8ml of culture medium to the flask and transfer the resulting suspension into a 15ml tube.
4. Centrifuge at 400g for 5 minutes.

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[4]It is important to leave the fibroblasts undisturbed for as long as possible whilst the cells are growing out of the tissue section.
[5]We usually isolate the HUVEC first, which opens and empties the vien, making it easier to cut along.
[6]Fast-growing smooth muscle cells in the secretory phenotype are isolated and expanded in this medium. Slow-growing contractile smooth muscles cells can be generated in vitro by culturing the 'secretory' smooth muscles for 72h in Promocell SMC medium containing only the 5% FCS (omit the growth factors from the complete medium). However, that medium is not compatible with endothelial co-culture (5).

5. Remove supernatant and resuspend the cell pellet in 0.5ml of culture medium and disperse by sucking them in and out of a pipette tip.
6. Make up to three volumes of culture medium and seed three flasks (see Note 7).
7. Repeat steps 1 through 5 to expand smooth muscle cells and fibroblasts for a minimum of four cycles before use in assays.

3.2.5 Freezing stromal cells—

1. Repeat steps 1 through 4 from section 3.2.4.
2. Add 3mls of ice cold DMSO:FCS (1:9 ratio) per 75cm^2 culture flask.
3. Add 1ml into an ice cold cryovial (Nalgene).
4. Store in −20°C for 2h.
5. Transfer to −80°C overnight.
6. Transfer to liquid N_2 until future need.
7. To use, thaw the cryovial rapildy at 37°C and transfer the 1ml contents into 5ml of cold medium (chose appropriate medium for different stromal cells).
8. Centrifuge at 400g for 5minutes.
9. Remove supernatant and resuspend the cell pellet in 4ml of culture medium and transfer to a T25 flask.

3.4 Establishing endothelial-stromal cell co-cultures on filters (see Note 8)

Depending on the type of assay, endothelial cells will be seeded inside the filter (inner surface) and stromal cells on the outside (outer) surface, or vice-versa. For static assays and for the parallel-plate flow chamber which takes cut out 6-well filters, seeding of the endothelial cells is on the inner surface, while for the flow chamber which takes intact 24-well inserts, seeding of the endothelial cells is on the outer surface. Whichever assay is employed, the stromal cells are seeded first.

3.4.1 Establishing stromal cell co-cultures—

1 Trypsinise a single flask of T75 of stromal cells as in section 3.2.4 and suspend cells in 8ml (see Note 9).

2a For use in static assays or phase contrast chamber, invert the filter in a sterile box and carefully seed stromal cells onto the outer surface of the inverted filter (200μl on a 24-well filter; 500μl on a 6-well filter). Incubate at 37°C for 1h, after which the filter is re-inverted and placed into wells containing culture medium.

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[7]From primary smooth muscle cell cultures, we typically expand to passage 4 before freezing in aliquots equivalent to one T25 flask. With primary fibroblasts, of four T25 from a divided tissue sample, one would be split three ways and passaged further, and three frozen in liquid nitrogen for later expansion. Experiments would typically done with cells between passage 4 and 10.

[8]Some studies on transendothelial migration have pre-coated the Transwell filters with collagen or fibronectin (FN) (10-13). It has been suggested that this coating increases the percentage of leukocyte migration. However, comparing uncoated filters with FN-coated filters (either coated before the assay with 20μg/ml human plasma FN (Sigma) or bought pre-coated with 170-200μg/ml FN from BD), we found no significant differences in the percentages of neutrophils transmigrating. We have not studied the effects of pre-coating on lymphocyte adhesion and migration.

[9]Typically we seed 2.5×10^4 fibroblasts or 1×10^5 smooth muscle cells in 200μl on 24-well filters and 30 times as many fibroblasts (7.5×10^5) in 500μl on 6-well filters.

2b For use in the fluorescence chamber, seed 200μl of stromal cells on the inner surface of the 24-well filters in their wells.

3 After 24h, trypsinise a single flask of HUVEC as in section 3.2.4. This will seed 4 6-well filters or 20 24-well filters (see Note 10).

4 Resuspend in 8ml of medium (see Note 11).

5a For use in static assays or phase contrast chamber, aspirate the medium from the upper chamber and seed 2ml of HUVEC to each filter (inner surface) for 6-well format or 200μl in 24-well format.

5b For use in the fluorescence chamber, seed HUVEC onto the outer surface as in 2a.

6 Culture endothelial cells with stromal cells for 24h.

7 Treat with cytokines if desired (see Note 12).

3.5 Adhesion and migration of lymphocytes through co-cultures on 3.0μm pore filters under static conditions (Fig 4)

Below we describe the volumes required when using 24-well filters; to use 12-well or 6-well filters the medium and cell numbers added must be scaled up accordingly.

1. Remove cytokine containing medium from the upper and lower chamber
2. Add 700μl of fresh M199+BSA to the lower chamber and 200μl of PBL or chosen lymphocyte sub-type (2×10^6cells/ml in M199+BSA) to the upper chamber (see Note 13).
3. Allow the PBL to settle, adhere and migrate (see Fig 4) at 37°C for desired time (typically 24h) (see Note 14).
4. Stop the experiment by transferring the filter into a fresh well.
5. Transfer the PBL from the upper chamber (above filter) into a fresh well.
6. Wash the upper chamber twice with 200μl of M199+BSA, and add washouts to the upper chamber samples. These represent the non-adherent PBL.
7. Retrieve cells from the original lower chamber, rinse out with 300μl of M199+BSA and pool with retrieved cells. Examine well microscopically to ensure that all cells are removed and wash further if necessary (see Note 15). The pooled

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[10]Cells from one confluent 25cm^{2}flask of HUVEC, resuspended in 8mls, will seed the inside of four 6-well filters (2mls per filter). Alternatively, one 25cm^2 flask can be resuspended in 4mls and used to seed the inner or outer surfaces of twenty 24-well filters (200μl per filter). Both produce a confluent monolayer within 24 hours.

[11]Our standard medium for growing HUVEC contains hydrocortisone. However, this can alter the inflammatory response induced by fibroblasts (3). It is important to consider whether the growth factors and corticosteroids added to medium alter the behaviour of the different cells. In this case, we withdraw hydrocortisone from the culture medium used for co-culture. In some experiments we attempted to co-culture HUVEC with SMC in the contractile phenotype. This required culture in the absence of growth factors and with only 5% FCS in the Promocell SMC medium. However, this medium was unsuitable for endothelial cell culture (2).

[12]In studies of lymphocyte adhesion and migration, we have stimulated HUVEC with TNF (100U/ml), IFN (10ng/ml) or both for 24h prior to assay. For endothelial-fibroblast co-cultures, we have tested how different fibroblasts modulate response to the combined cytokines. In studies with endothelial-smooth muscle cell co-cultures we use TNF over a range of concentrations for 24h (2).

[13]We have used PBL but assessed the content of different sub-populations in the added cells and those that transmigrated using flow cytometry. In this way, for example, migration of CD4+ and CD8+, naive or memory cells can be compared by choice of appropriate fluorescently-labelled antibodies.

[14]Lymphocytes are slower at migrating through filters than neutrophils (14-16). Whilst optimising this protocol we analysed lymphocyte migration at 2, 4 and 24h for unstimulated HUVEC and after stimulation with various cytokine combinations. Transmigration was very low at the early time points and increased significantly with time. We routinely use a 24h period for lymphocytes and 2h period for neutrophils.

[15]If lymphocytes adhere to the bottom of the well, it can be pre-coated with the non-adhesive substrate polyHEMA (17,18).

samples represent those cells that had migrated through both endothelial and stromal layers.

8. Count the ‘non-adherent’ and ‘transmigrated’ samples using a Coulter Counter (see Note 16) or haemocytometer.
9. Fix the filter in 2% isotonic glutaraldehyde containing at 1ug/ml bisbenziminde for 15 minutes in the dark and then wash four times in PBS.
10. Cut the filter out using a scalpel, directly onto a microscope slide and mount with anti-fade agent (e.g. DABCO; Sigma).
11. Using a fluorescence microscope with UV illumination and 40x objective, focus on the nuclei of the HUVEC. Move the focus down through the filter (a distance of ~10μm) until transmigrated lymphocytes adherent to the back of the filter come into view and count these cells.
12. These cells represent those which crossed the endothelium and filter, but were retained by the stromal cells. Their number can be added to the counts from the lower chamber to give the number of lymphocytes that migrated through endothelial cells and filter.
13. All counts should be expressed as a percentage of those originally added.
14. From this data, the percentage of adherent cells, the percentage transmigrated (below the filter and in the lower chamber), and the percentage held by the stromal cells can be determined.

3.6 Flow-based assay of lymphocyte adhesion and migration

We have described two different flow chambers. Using 0.4μm pore filters, the fluorescence chamber allows the analysis of lymphocyte recruitment from flow (capture, rolling and firm adhesion). The phase contrast chamber is suitable for visualising lymphocyte recruitment and additionally allows analysis of migration on, through and under the endothelial monolayer. If 3μm pore filters are used, then migration through the filter into the stromal layer can also be recorded. In our experience, this is a slow process for lymphocytes, but much faster for neutrophils.

3.6.1 Setting up the flow assay—

1. Assemble flow system without flow chamber attached (Fig 3). The electronic valve has a common output, and two inputs, from ‘Wash reservoir’ and ‘Sample reservoir’, which can be selected by turning electronic valve on and off.
2. Fill wash reservoir with PBSA and rinse through all tubing, valves and connectors with PBSA, ensuring bubbles are displaced (e.g., using syringe attached to 3-way tap for positive ejection). Fill sample reservoir with PBSA and rinse through valve and attached tubing. Prime downstream syringe and tubing with PBSA and load into syringe pump. All tubing must be liquid-filled to ensure prompt starting and stopping of flow.

3.6.2 Assembling and connecting the flow chamber for fluorescence microscopy (Fig 1)—

[16]There are alternative methods of analysing lymphocyte counts including pre-labelling with fluorescent dyes or radio-isotopes. BD Biosciences supply a Transwell filter which has a patented light-tight PET membrane that efficiently blocks the transmission of light within the range of 490-700 nm (www.bdbiosceinces.com). Using this system, the number of transmigrated fluorescent cells beneath the filter can be analysed using a fluorescence plate reader during the assay.

1. Align the glass coverslip and large silicon gasket on the top parallel plate, lower the bottom parallel plate onto the gasket and secure with metal screws.
2. Insert the complete 24-well filter into the machined receiving slot in the bottom parallel plate. The endothelial side of the filter aligns with the bottom plate forming a sealed base to the flow channel, butting onto the silicon gasket.
3. Place the small rubber gasket and Perspex plate over the base of the filter and secure into place with metal screws.
4. Connect Portex Blue Line Manometer connecting tubing (Portex Ltd, UK) into the inlet and outlet holes in the sides of the bottom plate.

3.6.3 Assembling and connecting the phase contrast flow chamber (Fig 2)—

1. Gently place the 6-well filter onto the centre of the 75×26mm glass coverslip; stromal cells on the outer surface of the filter in direct contact with the coverslip.
2. Using a new scalpel blade (type 10A), carefully cut out the filter.
3. Smooth a section of parafilm on a glass microscope slide, and cut round to form a gasket (75×26mm). Cut a slot 20×4mm to form the flow channel using an aluminium template (Fig 2)
4. Place the parafilm gasket over the coverslip; with the flow channel over the filter.
5. Put the glass coverslip into the milled recess in the bottom Perspex plate of the flow chamber and place the flow channel (top Perspex plate) over the endothelial cell surface (Fig 2).
6. Screw the top and bottom Perspex plates together.
7. Connect Portex Blue Line Manometer connecting tubing (Portex Ltd, UK) into the inlet and outlet holes in the top Perspex plate.

3.6.4 Perfusing cellular suspension and recording behaviour—

1. Place the flow chamber onto microscope stage and start flow by turning on syringe pump in withdrawal mode, with electronic valve and 3-way tap in position to allow delivery of PBSA from wash reservoir.
2. Wash out culture medium and locate the endothelial surface using phase-contrast (or bright-field microscopy if subsequent observations are with fluorescent lymphocytes).
3. Adjust flow rate to that required for assay. To obtain a given wall shear rate or stress, the flow rate (Q) will depend on the flow channel dimensions (see Note 17).
4. Perfusion of cells is typically at a flow rate Q=0.395ml/min for the fluorescence flow chamber (where the channel depth and width are 100μm and 5mm respectively) or Q=0.099ml.min for the phase contrast flow chamber (where the channel depth and width are 133μm and 4mm respectively). These are equivalent to a wall shear rate of $140s^{-1}$ and wall shear stress of 0.1Pa (=$1dyn/cm^2$), similar to those found in a post-capillary venules.
5. Load isolated cells into sample reservoir and allow to warm for 5 minutes.
6. Switch the electronic valve so cell suspension is drawn through microslide
7. Deliver timed bolus (e.g., 4 minutes). Typically, flowing cells will be visible after about 30s, the time required to displace dead volume in valve and tubing.

8. Switch electronic valve so that PBSA from wash reservoir is perfused. Again, 30-60s will be required before all cells have been washed through the flow chamber or microslide.

9. Video recordings can be made as desired during inflow and washout of cells. Typically, a series of fields may be recorded along the centreline of the chamber during inflow (e.g., 6 fields recorded for 20s each during the last minute of the bolus), for off-line analysis of the behaviour (e.g., rolling or stationary adhesion) of the cells. Another series can be made after 1 minute washout (when the bolus is complete) for analysis of the numbers of adherent cells and their behaviour. Fields may be recorded at later times (e.g., after a further 5 and 10 minutes) to assess progress of migration (e.g., through the monolayer or filter), and/or a field can be recorded continuously for 5-10min to track individual cells and assess velocity of migration. At later time points, recordings can be made of cells beneath 3μm pore filters (by focussing 10um down) and beneath the stromal cell layer.

10. If a defined timing protocol is developed, digital images, or sequences of digital images could be recorded instead of video images. The continual recording of the latter gives flexibility in analysis.

11. Data analysis is carried out off-line.

3.6.5 Analysis of cell behaviour from video recordings—

1. Make recordings of a microscope stage-micrometer oriented parallel and perpendicular to the flow. Use this to calibrate the size of video field observed on the monitor during playback and the image analysis software.

2. To quantify the numbers of adherent cells and their behaviour, digitise a sequence of images 20 at 1 second intervals from recordings made at the desired times.

3. When played in a loop, cells can be distinguished which are rolling (circular phase bright cells tumbling slowly at ~1-10μm/s over the surface) or stabley adherent on the endothelial surface (phase bright cells typically with distorted outline and migrating slowly on the surface) or transmigrated cells (phase-dark spread cells migrating under the HUVEC). Non-adherent cells will only be visible as blurred streaks. Migrated cells beneath the filter and beneath the stromal cell layer appear phase bright cells with a distorted shape. When using the fluorescence system – all recruited cells appear bright, with rolling cells being spherical and stabley adherent/ migrating cells typically being distorted in shape.

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[17]The flow rate (Q) required to give a desired wall shear rate (γ_w in s^{-1}) or wall shear stress (τ_w in Pascal, Pa) is calculated from the internal width (w) and internal depth (h) of the flow channel and the viscosity (n) of the flowing medium using the formulae,

$$\gamma_w = (6.Q) / \left(w.h^2\right)$$

$$\tau = n.\gamma$$

For the fluorescence flow chamber, w and d are 5mm and 100μm respectively.
For the phase contrast flow chamber, w and d are 4mm and 133μm respectively, although the depth varies slightly from parafilm gasket to gasket.

4. Count the cells present on a stop-frame video field at the start of a sequence, and then play the loop to assign them as rolling, stationary or transmigrated. Repeat and average counts for the series of sequences recorded at a given time.

5. Convert counts of total adherent cells (rolling + stationary + migrated) to number/mm^2 from the known field dimensions. Divide this by the number of cells perfused (in units of 10^6 cells) to obtain number adherent/$mm^2/10^6$ perfused. The number perfused is calculated by multiplying the concentration of the suspension (usually 10^6/ml) by the flow rate by the duration of the bolus (e.g., 4 minutes). This normalisation allows correction for changes in conditions (bolus duration, cell concentration, flow rate) between experiments, and effectively calculates an efficiency of adhesion.

6. Express the numbers of cells rolling, stationary adherent or transmigrated as percentages of the total adherent cells. When location below the filter is analysed, counts should be added to the total, and the percentages in this location calculated.

7. Analysis at different times (e.g., after 1, 5 or 10 minutes of washout) can be used to quantify the progress of migration through the different layers (endothelium, filter and stromal cells) or any changes in behaviour.

8. To measure rolling velocity, mark the leading edges of a series of cells to be followed and move to second captured frame. Remark the leading edges and record the distance moved. Repeat through the 10 second sequence. This will yield data for position versus time. Velocity for each cell can be averaged over the observation time, and estimates of variation in velocity made if desired.

9. To measure migration velocity in extended video sequences, images are digitised at one minute intervals over 5-10 minutes. The cells are outlined and the positions of their centroids recorded at each minute. The changes in positions are used to calculate the distances migrated in each minute. The average velocities can be calculated from the sequence.

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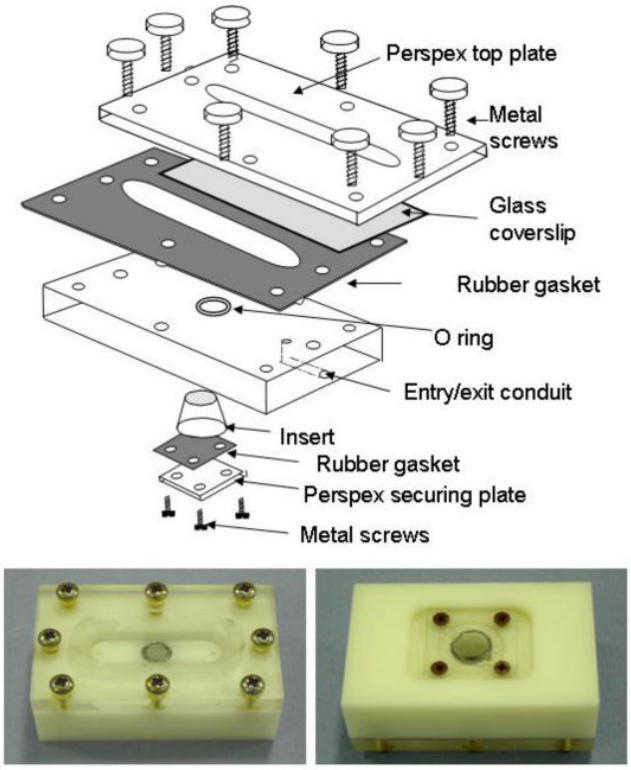

Fig 1.

Fluorescence parallel plate chamber. Two parallel perspex plates are separated by a glass coverslip (5.5 × 2.6mm) and a non compressible gasket cut from silicon sheet (Esco rubber, 250μm thick; Bibby Sterilin Ltd, Stone, UK) with a flow channel of 41 × 6mm and depth of 250 μm cut in it. The plates are held in place by hand-tightened metal screws. Filter inserts are placed into a machined receiving slot of a complementary size for the 24-well insert within the lower parallel plate. The insert forms a sealed base to the flow channel and is held in place by a smaller rubber gasket and perspex plate, held in place with metal screws. The surface of the filter is viewed in an upright microscope. The depth of the back plate holding the filter insert is too great to allow focussing of the transmitted light condenser, so that phase contrast images of high quality cannot be obtained. The surface is thus viewed during experiments using incident light illumination and fluorescence.

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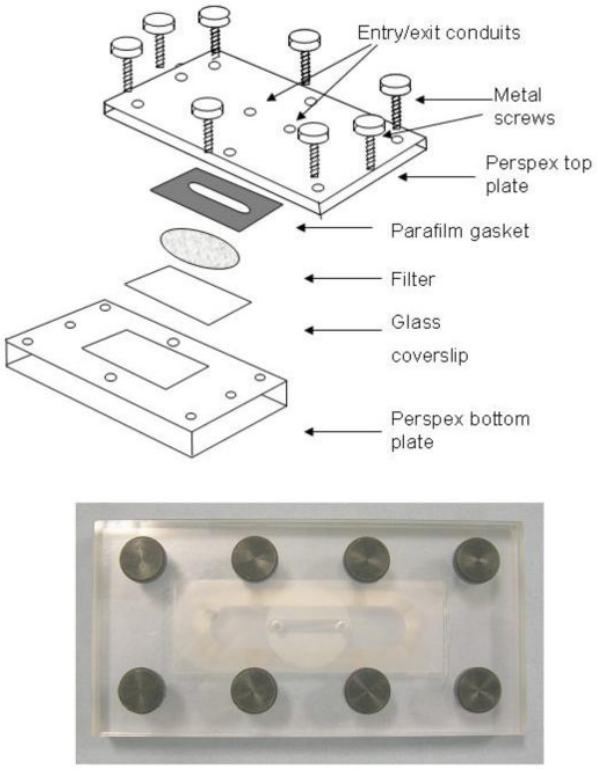

Fig 2.

Phase contrast parallel plate chamber. Cells are seeded onto six-well filters, which are cut out onto the glass coverslip (76×26mm). The filter and coverslip are covered with a parafilm gasket of the same size, with a flow channel of 20 × 4mm and depth of 133μm cut in it. These are placed on a perspex base-plate with a shallow matching recess milled into it and a viewing slot cut in it. The upper Perspex plate has inlet and outlet holes positioned to match the flow channel formed by the gasket slot, allowing liquid to be perfused over the endothelium. The plates have matching holes (threaded in the lower plate) to allow them to be clamped together with hand screws. The parafilm gasket is cut afresh for each coverslip, using a thin aluminium sheet template, 76×26mm, with 20 × 4mm slot machined in it.

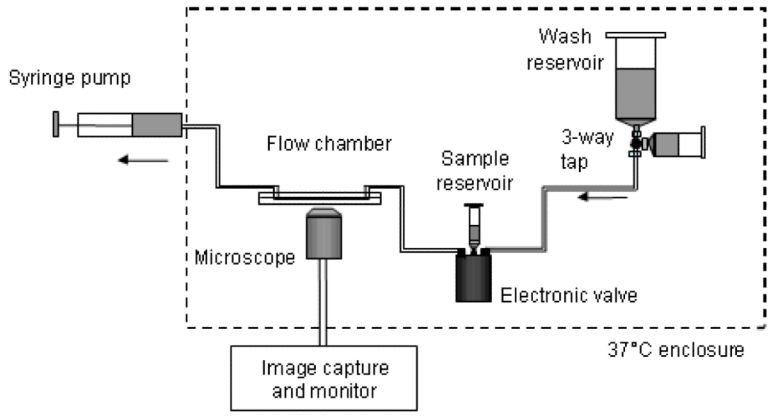

Figure 3.

Schematic representation of assembled flow system. The parallel plate flow chamber was incorporated into a perfusion system mounted on the stage of a phase-contrast and fluoresencemicroscope enclosed in a Perspex chamber at 37°C. It was connected by flexible silicon tubing to a Harvard withdrawal syringe pump at one end, or an electronic switching valve at the other. A suspension of purified leukocytes or cell free wash buffer was perfused through the chamber, typically at a constant wall shear stress of 0.1Pa. Images from the microscope were captured using CCD video camera and video tape and subsequently digitised for analysis, or captured using digital camera straight to computer.

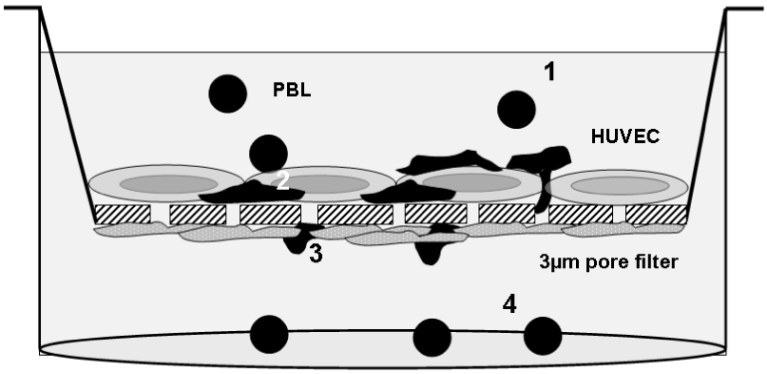

Figure 4. Schematic representation of the Transwell assay

Lymphocytes (2×10^6cells/ml) are added into the upper chamber and allowed to interact with the TNF-stimulated endothelial cells (HUVEC). The lymphocytes either remain non-adherent or become attached to the surface of the HUVEC or migrate through them (2 = white). The lymphocytes may migrate through the filter and either remain adherent to the basal surface among the fibroblasts (3) or fully migrate into the lower chamber of the tissue culture plate (4). Counting of cells retrieved from the upper and lower chambers determines the percentage of lymphocytes that are non-adherent (1) or that fully transmigrate (4). Counting of stained cells below the filter using fluorescence microscopy allows analysis of those that transmigrated but were retained by fibroblasts (3). Total transmigratio = (3) + (4).